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## Research paper

# Fasting unmasks differential fat and muscle transcriptional regulation of metabolic gene sets in low versus normal birth weight men



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## ABSTRACT

**Background:** Individuals born with low birth weight (LBW) have an increased risk of metabolic diseases when exposed to diets rich in calories and fat but may respond to fasting in a metabolically preferential manner. We hypothesized that impaired foetal growth is associated with differential regulation of gene expression and epigenetics in metabolic tissues in response to fasting in young adulthood.

**Methods:** Genome-wide expression and DNA methylation were analysed in subcutaneous adipose tissue (SAT) and skeletal muscle from LBW and normal birth weight (NBW) men after 36 h fasting and after an isocaloric control study using microarrays.

**Findings:** Transcriptome analyses revealed that expression of genes involved in oxidative phosphorylation (OXPHOS) and other key metabolic pathways were *lower* in SAT from LBW vs NBW men after the control study, but paradoxically *higher* in LBW vs NBW men after 36 h fasting. Thus, fasting was associated with down-regulated OXPHOS and metabolic gene sets in NBW men only. Likewise, in skeletal muscle only NBW men down-regulated OXPHOS genes with fasting. Few epigenetic changes were observed in SAT and muscle between the groups.

**Interpretation:** Our results provide insights into the molecular mechanisms in muscle and adipose tissue governing a differential metabolic response in subjects with impaired foetal growth when exposed to fasting in adulthood. The results support the concept of developmental programming of metabolic diseases including type 2 diabetes.

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## 1. Introduction

Individuals born with low birth weight (LBW) have increased risk of obesity [1] and type 2 diabetes (T2D) [2,3] later in life. Young adults born with LBW exhibit increased abdominal fat mass [4], increased whole body lipolysis [5], and reduced expression of key insulin signalling proteins in subcutaneous adipose tissue (SAT) and skeletal muscle

[6,7]. These changes may all contribute to the increased risk of T2D in LBW individuals, especially if subjected to diets rich in calories and fat. Accordingly, we have shown that LBW subjects exposed to a 5-day high-fat overfeeding challenge were more prone to develop peripheral insulin resistance than normal birth weight (NBW) controls [8]. Furthermore, appetite regulating hormones including leptin and PYY were lower in LBW compared with NBW subjects after overfeeding [8,9].

Caloric restriction, including fasting, has been associated with improved health and survival in several animal and human studies [10]. Nevertheless, there is limited knowledge of whether people respond to fasting in a differential manner, and the extent to which this might influence their risk of cardiovascular and metabolic diseases. In a recent study we showed that healthy young men born with LBW have a

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## Research in context

### Evidence before this study

Caloric restriction, including fasting, has been associated with improved survival and health in several animal and human studies. However, the molecular mechanisms contributing to this remains to be dissected in humans. Additionally, individuals born with a low birth weight (LBW) have an increased risk of obesity, diabetes and cardiovascular disease when exposed to diets rich in calories and fat and they may benefit from fasting. In 2015, we showed that healthy young men born with LBW have a more energy conserving phenotype after 36 h of fasting than matched normal birth weight (NBW) control subjects, including lower energy expenditure, lower fat oxidation and lower triglyceride levels. To clarify whether LBW individuals have a different molecular response to fasting than NBW individuals, we carried out a study of genome-wide transcription and DNA methylation in human adipose tissue and skeletal muscle from 37 young healthy men born with LBW or NBW after 36 h of fasting and after an isocaloric control diet.

### Added value of this study

This study provides the first molecular evidence that fasting is associated with a downregulation of oxidative phosphorylation (OXPHOS) and metabolic gene sets in NBW men, whereas LBW men displayed unchanged levels of metabolic gene sets in adipose tissue with fasting. Moreover, we found that expression of genes involved in OXPHOS and key metabolic pathways were *lower* in adipose tissue, and partly also in muscle, from LBW vs NBW men after control conditions, but paradoxically *higher* in LBW vs NBW men after 36 h fasting. The lower expression of OXPHOS and other key metabolic gene sets in adipose tissue during isocaloric control conditions was further validated in an independent cohort of LBW and NBW individuals. DNA methylation alterations could not explain the differences in mRNA response to fasting between the LBW vs NBW men in the current study.

### Implications of all the available evidence

This study provides novel human transcriptomic and epigenetic data from human adipose tissue and muscle after prolonged fasting. Our results support an impact of foetal growth on the regulation of the transcriptional response to fasting in adulthood, which may contribute to the predisposition of metabolic diseases in LBW individuals. Overall, our data explain why some people may benefit from fasting with an improved metabolic phenotype while other will not.

more energy conserving phenotype after 36 h of fasting than matched NBW control subjects, including lower energy expenditure, lower fat oxidation, and lower insulin and triglyceride levels [11]. These data strongly support the so called 'thrifty phenotype hypothesis' and the assumption that LBW individuals in adulthood may have an improved tolerance to - or cope better with - fasting/energy restriction that was shaped in utero. Some studies suggest that the adult 'memory' of an adverse foetal development can be directed to epigenetic modifications e.g. DNA methylation and less epigenetic changes took place in muscle of LBW versus NBW men when exposed to a short-term high-fat diet [11–15]. In another study, we found reduced epigenetic flexibility in adipose tissue from LBW subjects in the genes encoding leptin and adiponectin in response to fasting [16]. However, the molecular

alterations that cause insulin resistance in adipose tissue and skeletal muscle from LBW individuals are still not well documented.

We hypothesized that fasting might unmask transcriptional and/or epigenetic alterations in insulin sensitive tissues from healthy individuals who were born with LBW. Such transcriptional and epigenetic differences may contribute to metabolic differences seen in LBW vs NBW subjects and predispose to T2D and obesity. To address these questions, we applied an unbiased array approach to determine genome-wide gene expression and DNA methylation patterns in subcutaneous adipose tissue (SAT) and skeletal muscle from healthy young men born with LBW and matched NBW controls after 36 h fasting and after a control study (12 h overnight fasting). We first tested whether there are differences between the birth weight groups, both after 36 h fasting and after the control study. Secondly, we analysed differences between the fasting intervention and the control study in both birth weight groups separately. We performed these analyses for physiological parameters as well as for gene expression and DNA methylation in SAT and skeletal muscle.

## 2. Materials and methods

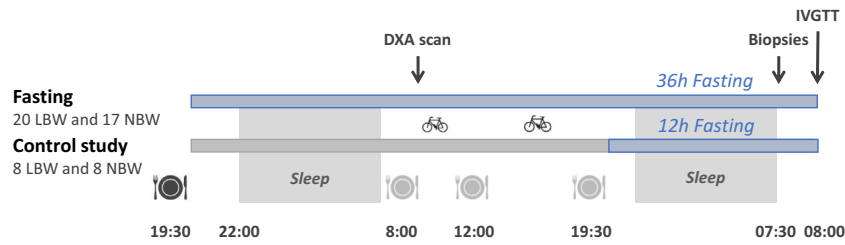
### 2.1. Study design

Healthy, young (22–27 years) Danish men born at term (week 39–41) were recruited from the Danish National Birth Register [11]. SAT and skeletal muscle biopsies were excised from 37 men, whereof 20 born with a LBW (lowest 10th percentile) and 17 age- and BMI-matched controls born with a NBW (50th–75th percentile) (Table S1). Exclusion criteria were diabetes in the family in two generations, physical exercise > 10 h/week, BMI > 30 kg/m<sup>2</sup> or use of medication known to affect metabolism [11].

All participants were subjected to 36 h of fasting (*fasting study*), and after 2–4 months, 8 of the LBW and 8 of the NBW men participated in a *control study* where the subjects received standardized meals and biopsies were excised after an overnight (12 h) fast [16] (Fig. 1). One LBW and two NBW men did only participate in the collection of overnight fasting samples and biopsy excision after 3 days of standardization by control diet and they did not participate in further clinical examinations during the control study [16]. Three days prior to the fasting and control studies, all participants received standardized, pre-cooked meals with fixed calories (10 MJ/day) containing 35% fat, 15% protein and 50% carbohydrate, and abstained from exercise, alcohol and soft drinks [11]. The participants received a standardized meal at 7:30 p.m. after arriving at the laboratory and fasted thereafter. They were instructed to go to sleep at 10:00 p.m. Water ad libitum was allowed, and smokers were allowed to use a 10 mg Nicorette® inhaler (McNeil). Individuals spent two 10-min sessions on an ergometer bicycle during the first day to avoid inactivity. On the first day of the control study, participants received standardized meals at 8:00 (breakfast), 12:00 (lunch), 15:00 (snack) and 19:30 (dinner) (Fig. 1). On the second day between 7:00 and 7:30, after 36 h (fasting study) and 12 h (control study) fasting respectively, abdominal SAT and skeletal muscle (*vastus lateralis*) biopsies were excised. Short after, a 180-min intravenous glucose tolerance test (IVGTT) was initiated. Written informed consent was obtained before the study from all participants. The local ethics committee approved the study (HD-2008-127) and the protocol was in accordance with the Helsinki II declaration.

### 2.2. Clinical examinations

The fasting study and the control study were carried out with identical study settings, apart from one LBW and two NBW men of the control study who, after 3 days of standardized meals, only participated in the collection of tissue biopsies and blood samples after an overnight fasting. Clinical examinations of this study have been described in detail previously [11,16]. Anthropometric measurements were determined



**Fig. 1.** Study design. The fasting study (36 h fasting; 20 LBW and 17 NBW) and the control study (standardized meals followed by 12 h overnight fasting; 8 LBW and 8 NBW men) was separated by a period of 2–4 months. The time indicates when different interventions (sleep, meals, biopsy excision and IVGTT) were performed during the first, second and third day of the study regimen. The grey coloured plate, knife and fork images represent the standardized meals in the control study. IVGTT, intravenous glucose tolerance test; LBW, low birth weight; NBW, normal birth weight.

the first morning. Intravenous blood samples were drawn from the antecubital vein at 08:00 and 14:00 the first day, and hourly from 20:00 until 7:59 the following morning to sample for glucose, insulin and non-esterified fatty acids. Tissue biopsies were excised under local anaesthesia with Xylocain (AstraZeneca, Cambridge, UK) using a Bergström needle with suction, snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Energy expenditure (EE) and substrate oxidation rates were examined with indirect calorimetry for 30 min at baseline (control study) and after 31 h and 34 h of fasting (fasting study; Deltatrac monitor, Datex instruments). The respiratory quotient (RQ) was calculated as  $\text{CO}_2$  expired/ $\text{O}_2$  consumed, and net lipid and glucose oxidation rates were calculated using the non-protein RQ [17].

### 2.3. Genome-wide gene expression analyses in SAT and skeletal muscle

Total RNA was extracted from 52 SAT biopsies (20 LBW and 16 NBW after fasting, 8 LBW and 8 NBW after control study) using the miRNeasy Kit (Qiagen, Hilden, Germany) and subsequently purified from small RNA molecules with the RNeasy MinElute Cleanup Kit (Qiagen). Similarly, RNA from 52 skeletal muscle biopsies (20 LBW and 16 NBW after fasting, 8 LBW and 8 NBW after control study) was extracted with TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). RNA quality was assessed with the Agilent 2100 Bioanalyzer system (Agilent Technologies, Basel, Switzerland). Genome-wide expression of 28,838 annotated transcripts representing 18,229 unique genes was analysed in all RNA samples using the GeneChip Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) following Affymetrix standard protocol.

### 2.4. Genome-wide DNA methylation analyses in SAT and skeletal muscle

DNA extracted from 51 SAT biopsies (19 LBW and 16 NBW after fasting, 8 LBW and 8 NBW after control study) with the QIAamp DNA Mini Kit (Qiagen) and 50 skeletal muscle biopsies (18 LBW and 16 NBW after fasting, 8 LBW and 8 NBW after control study) with the DNeasy Kit (Qiagen) was used for DNA methylation analysis using Infinium HumanMethylation450 BeadChips (Illumina, San Diego, CA, USA). Paired samples (control and fasting) were placed on the same chip, and samples from LBW and NBW individuals were evenly distributed.

### 2.5. Bioinformatics and statistical analysis

Gene expression data were background corrected and quantile normalized with robust multi-array average (RMA) using Bioconductor [18,19]. Subsequently, the array data was analysed with unpaired (LBW vs NBW) and paired (fasting vs control) Student's *t*-tests in R ([www.r-project.org](http://www.r-project.org)). Gene expression analyses in SAT were performed in all 37 individuals, but one NBW participant was only analysed at the control study due to lack of SAT RNA after the fasting study. Thus, 7 NBW and 8 LBW men were included in the paired analyses of the transcriptional response to fasting in SAT. In skeletal muscle, the

transcriptional response to fasting vs control study was analysed in 8 NBW and 8 LBW men. We applied false discovery rate (FDR) analysis to account for multiple testing, where  $\text{FDR} < 5\%$  ( $q < 0.05$ ) was considered significant.

For the DNA methylation data, methylation values were calculated from methylated and unmethylated allele intensity values and analysed as previously described [20]. ComBat was used for batch-correction [21] and  $\beta$ -values from 0 to 1 (0–100% methylation) were used to report the outcome. DNA methylation data was analysed with Wilcoxon tests (fasting vs control study) and two-sample Mann–Whitney *U* tests (LBW vs NBW). FDR corrections for DNA methylation were performed on subsets of genes included in this study. All *p*- and *q*-values presented are two-tailed.

### 2.6. GSEA pathway analysis

For the mRNA expression data, pathway analyses were performed on curated gene sets of KEGG (Kyoto Encyclopedia of Genes and Genomes) using Gene Set Enrichment Analysis (GSEA; <http://www.broadinstitute.org/gsea/>) [22,23]. For differences between LBW and NBW men, unpaired *t*-tests were applied on expression values after RMA normalization using Bioconductor [18,19]. For differences between fasting and control study, individual gene expression ratios and paired *t*-tests were used to create a gene list where each transcript got a rank based on the significance of the analysis. The GSEA analysis included 186 gene sets (size 9–349 genes/gene set). Results were sorted on normalized enrichment scores and  $q < 0.05$  was considered significant based on FDR. Pathways related to human diseases are excluded in figures and separated from the other pathways in tables due to their overlap with enriched genes in other pathways (i.e. OXPHOS and Parkinson's disease).

### 2.7. Validation cohort

In a previous study, 16 young healthy LBW men and 24 matched NBW men were exposed to 5 days of high-fat overfeeding (HFO; +50% calories, 60% of energy from fat) and an individualized weight-maintaining 3-day control diet (35% of energy from fat) in a randomized order separated by a washout period of 6–8 weeks [8]. Genome-wide gene expression was analysed using GeneChip Human Gene 1.0 ST arrays (Affymetrix) in SAT biopsies excised after an overnight fast [24]. Bioinformatics, *t*-tests and GSEA analyses were performed as in the fasting study.

## 3. Results

### 3.1. Physiological differences between LBW and NBW men as well as in their response to fasting

Physiological and metabolic differences between birthweight groups, and their response to fasting, was studied in all participants



after 36 h fasting (fasting study, 20 LBW and 17 NBW men) and for a subset of the participants after an isocaloric diet followed by an overnight (12 h) fast (control study, 8 LBW and 8 NBW men) (Fig. 1). Their clinical characteristics are presented in Table S1 and Table 1, respectively.

First, we compared physiological differences after an overnight fast between birth weight groups in all participants as well as in the subgroup included in the control study. The LBW and NBW individuals were matched for age and BMI, and there were no significant differences in their glucose, insulin or lipid profiles in neither all participants (Table S1; 20 LBW vs 17 NBW) nor in the subgroup that only participated in the control study (Table 1; 8 LBW vs 8 NBW). Moreover, in the control study, there were no significant differences in EE, RQ and glucose/fat oxidation between the LBW and NBW men, but EE and fat oxidation (FOX) tended to be lower in the subgroups of LBW vs NBW men (EE: 1411 vs 1562 kcal/24 h,  $p = 0.08$ ; FOX: 30.2 vs 41.4 mg/min,  $p = 0.12$ ; Table 1).

We then studied the physiological differences between birth weight groups after 36 h fasting in the full cohort. Here, the LBW men had significantly lower EE (1633 vs 1768 kcal/24 h,  $p = 0.046$ ), FOX (78.6 vs 91.4 mg/min,  $p = 0.02$ ) and triglyceride levels (0.78 vs 0.95 mmol l<sup>-1</sup>,  $p = 0.02$ ; Table S1) compared to NBW men. Glucose oxidation and levels of FFA, plasma glucose and serum insulin in blood were similar between groups after fasting.

Next, we investigated the physiological response to fasting by comparing the subjects included in both the fasting and the control study (Fig. 1). After fasting compared to after the control study, plasma glucose and serum insulin levels dropped drastically and the liver of both LBW and NBW men became more insulin sensitive based on the HOMA-IR index (Table 1). The EE increased significantly with fasting in the LBW men only ( $p = 0.02$ ). The decrease in glucose oxidation and increase in fat oxidation was significant in both groups, reflected by a significantly lower RQ after fasting (Table 1). Triglyceride levels dropped significantly in the LBW men only, whereas FFA levels increased >3-fold in both groups ( $p \leq 0.005$ ).

Together, there are physiological differences between the birth weight groups mainly seen after and in response to fasting.

### 3.2. Differences in expression of metabolic gene sets in SAT and skeletal muscle in LBW compared with NBW subjects after control and fasting studies

In the control study, gene expression in SAT and skeletal muscle was investigated in 8 LBW and 8 NBW subjects. After fasting, RNA from SAT and muscle was available for 20 LBW and 16 NBW subjects. We first tested if sets of related genes (KEGG pathways) had different expression in LBW versus NBW men by performing GSEA [23].

#### 3.2.1. SAT gene expression

After the control study, SAT displayed 27 significant gene sets with lower expression in LBW vs NBW men (Fig. 2; Table S2, sheet A). These include genes involved in oxidative phosphorylation (OXPHOS, Fig. 3A and B) and multiple other central metabolic pathways in carbohydrate, lipid and amino-acid metabolism. Remarkably and in contrast, after fasting, expression of 17 gene sets including several of these metabolic pathways were paradoxically increased in SAT from LBW vs NBW men (Fig. 2; Fig. 3A and B; Table S2, sheet B). Moreover, after the control study, six pathways involved in processing of genetic information were downregulated in SAT from LBW men (Fig. 2; Table S2, sheet A). We then tested whether the expression of individual genes was different in SAT of LBW vs NBW men after the control study and/or fasting. Some genes showed nominal differences in expression ( $p < 0.05$ ) between the groups but none were significant after correction for multiple testing (data not shown).

#### 3.2.2. Muscle gene expression

Similar to SAT, in the control study, we found lower expression of genes in the OXPHOS pathway in skeletal muscle from LBW vs NBW men, together with a reduction of folate biosynthesis (Fig. 4; Table S2, sheet C). In contrast, after fasting, muscle from LBW compared with NBW men displayed no significant differences in OXPHOS expression (Fig. 3A and C) and lower expression of three gene sets including the ribosome pathway and ECM receptor interaction (Fig. 4; Table S2, sheet D). Some individual genes had nominal differences in expression ( $p < 0.05$ ) in skeletal muscle from LBW vs NBW men after the control study and/or fasting but these were not significantly different after correction for multiple testing.

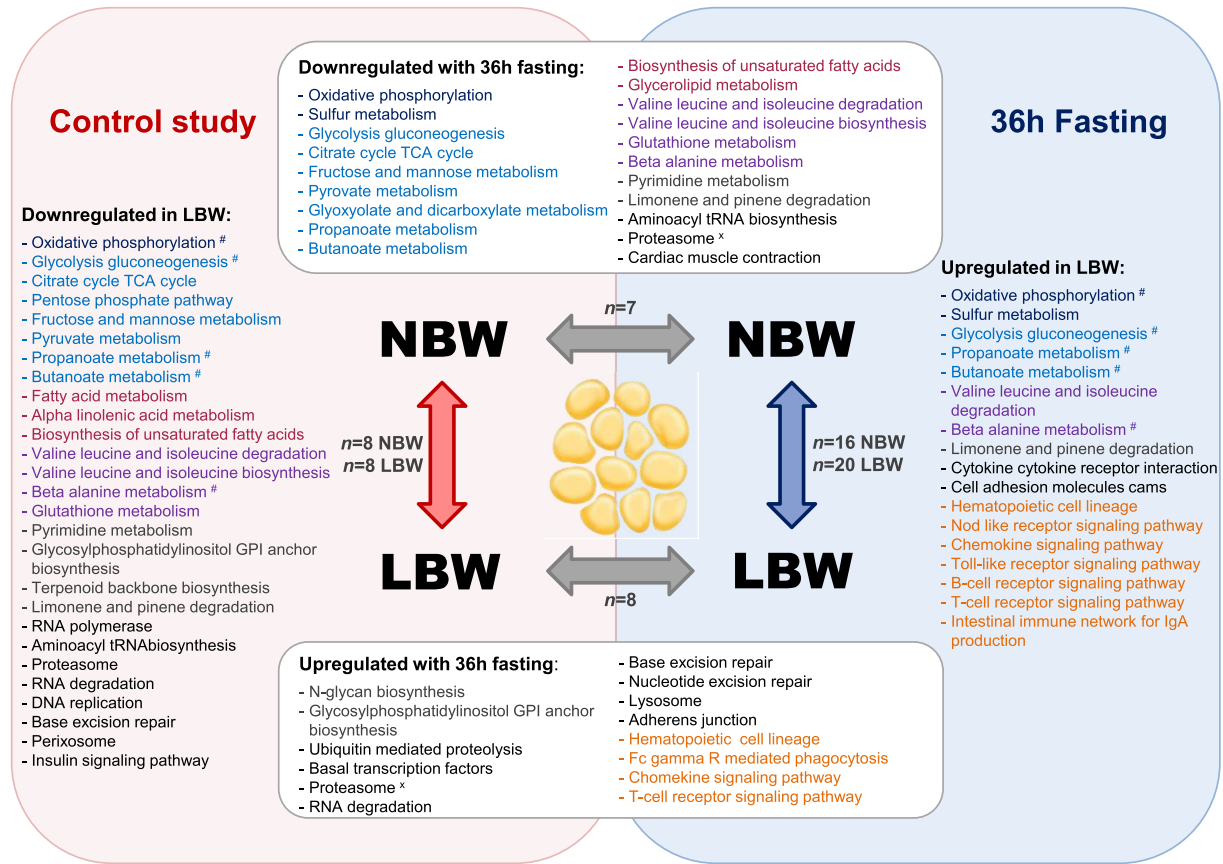
**Table 1**

Clinical characteristics of the LBW and NBW participants who were exposed to both 36 h fasting and the control study (12 h overnight fast).

Parameter	LBW (n = 8)	LBW (n = 8)	NBW (n = 8)	NBW (n = 8)
	Control study	Fasting	Control study	Fasting
<b>Body composition</b>				
Weight (kg)	71.8 ± 9.1	71.9 ± 9.6	80.3 ± 11.7	81.1 ± 11.6
BMI (kg/m <sup>2</sup> )	22.7 ± 2.7	22.7 ± 2.8	23.5 ± 4.0	23.7 ± 4.1
Body fat (%)	15.4 ± 5.7	15.9 ± 5.9	16.2 ± 5.5	16.7 ± 5.4
WHR	0.93 ± 0.04	0.90 ± 0.06	0.91 ± 0.05	0.90 ± 0.06
<b>Glucose and Insulin profile</b>				
Plasma glucose (mmol l/L)	4.9 ± 0.4	3.9 ± 0.3**	5.0 ± 0.3	3.9 ± 0.5**
Serum insulin (pmol l <sup>-1</sup> )	23.8 ± 7.9	12.2 ± 5.5*	21.1 ± 6.7	11.4 ± 3.1*
<b>Insulin sensitivity</b>				
HOMA-IR	0.75 ± 0.28	0.41 ± 0.17*	0.68 ± 0.23	0.38 ± 0.19*
<b>Indirect calorimetry measurements</b>				
EE (kcal/24 h) <sup>a</sup>	1411 ± 83	1550 ± 137*	1562 ± 182	1642 ± 195
RQ <sup>a</sup>	0.86 ± 0.03	0.78 ± 0.02**	0.85 ± 0.02	0.76 ± 0.03*
GOX (mg/min) <sup>a</sup>	116.7 ± 24.0	61.0 ± 21.9**	123.9 ± 22.4	46.3 ± 28.0*
FOX (mg/min) <sup>a</sup>	30.2 ± 11.6	69.2 ± 16.7**	41.4 ± 10.8	86.6 ± 22.0*
<b>Lipid profile</b>				
FFA (μmol l <sup>-1</sup> ) <sup>a</sup>	161 ± 54	582 ± 166**	173 ± 61	556 ± 213**
Total cholesterol (mmol l <sup>-1</sup> )	4.4 ± 0.5	4.4 ± 0.4	4.3 ± 0.8	4.3 ± 0.9
HDL cholesterol (mmol l <sup>-1</sup> )	1.27 ± 0.29	1.27 ± 0.31	1.05 ± 0.13	1.06 ± 0.22
Triglycerides (mmol l <sup>-1</sup> )	1.04 ± 0.25	0.80 ± 0.17*	1.07 ± 0.34	1.01 ± 0.26 #

Data are mean ± SD. Clinical data was obtained 1 h after biopsy excision after both the 36 h fasting and the control study intervention except for indirect calorimetry measurements that were obtained after 34 h fasting. Significant differences for paired comparisons between 36 h fasting and control study: \* $p \leq .05$ . \*\* $p \leq .005$ . Significant differences for unpaired comparisons between LBW and NBW men: # $p \leq .05$ .

<sup>a</sup> Data from 7 of the LBW and 6 of the NBW individuals only. EE, energy expenditure; RQ, respiratory quotient; GOX, glucose oxidation; FOX, fat oxidation. HOMA-IR was calculated from minimal modelling. Clinical data from the fasting intervention has been published previously [11].



**Fig. 2.** Key metabolic gene sets are differentially regulated in subcutaneous adipose tissue after 36 h fasting vs control study (horizontally) and in LBW vs NBW men (vertically). KEGG Metabolism pathways are coloured by subgroup: Energy (dark blue), Carbohydrate (light blue), Lipid (red), Amino acid (purple), Other amino acids (grey), Nucleotide, Glycan biosynthesis and Terpenoids and polyketides (grey). Also coloured: Immune system (Organismal system; orange). Human diseases pathways are not included in the figure. <sup>#</sup>Pathway lower in LBW vs NBW after control study and higher in LBW vs NBW after fasting. <sup>\*</sup>Pathway regulated in the opposite direction in response to fasting in LBW and NBW men. SAT, subcutaneous adipose tissue; LBW, low birth weight; NBW, normal birth weight.

The lower OXPHOS expression seen in SAT and muscle from LBW men are in line with what is seen in SAT and muscle from subjects with T2D and may predispose to diabetes [22,25]. The mRNA expression of individual OXPHOS genes were not statistically significant in LBW vs NBW men after correction for multiple testing, as described above.

### 3.3. Identification of transcription factor binding motifs of importance for differential gene expression in LBW vs NBW men

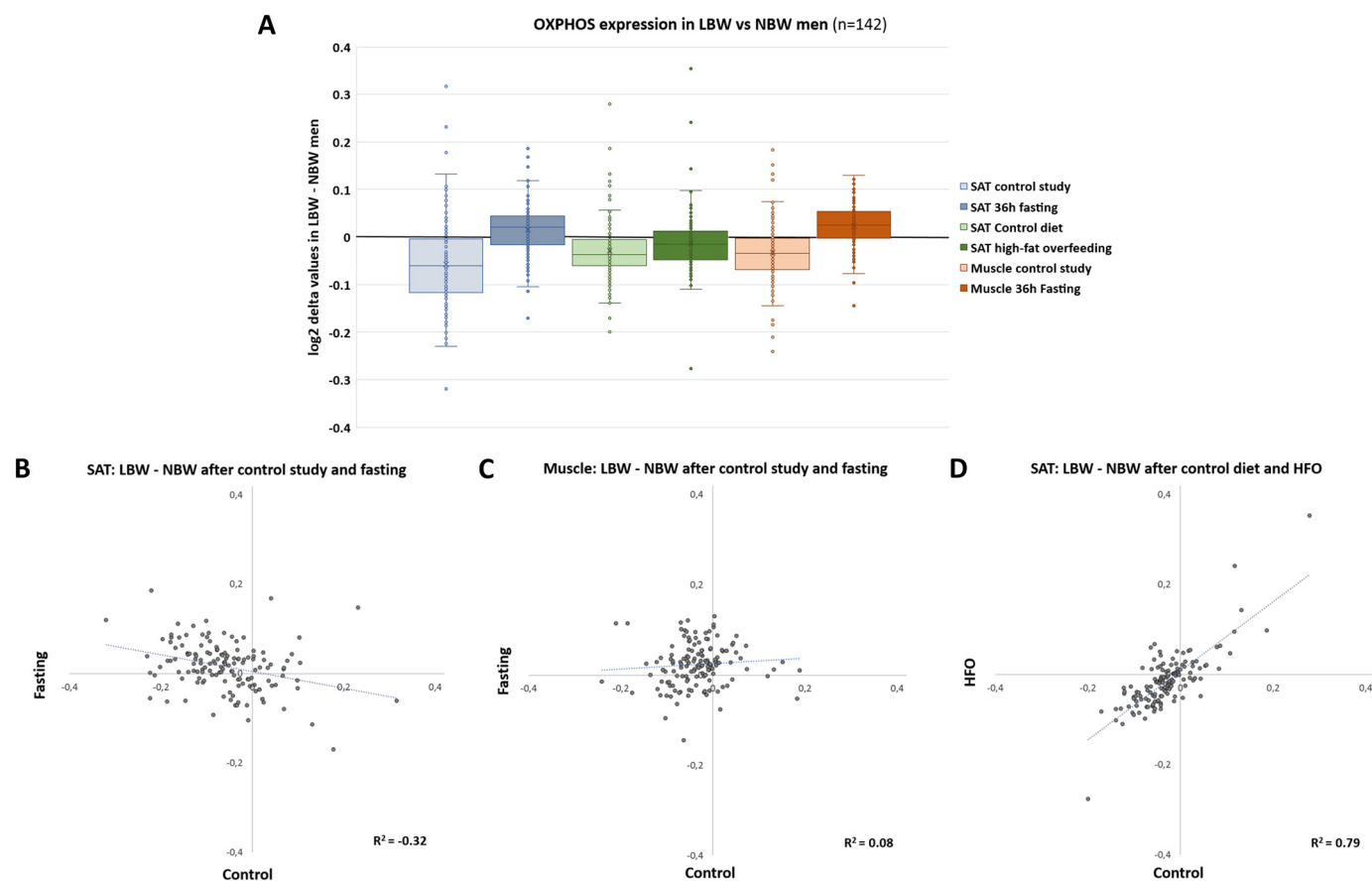
To identify transcription factors that may control the transcriptional differences in SAT and muscle between LBW and NBW subjects, we used PSCAN and JASPAR [26,27]. Here, we searched for overrepresentation of specific transcription factor binding sequences among the genes contributing to enrichment in significant GSEA pathways (Table S2, sheet A–D). Recognition sequences for 3 transcription factors, including KLF14, were significantly enriched in SAT based on enriched genes contributing to the 20 most significant KEGG pathways with lower expression in LBW vs NBW men after the control study (Table S3, sheet A), whereas the enriched genes in gene sets with higher expression in SAT from LBW men after fasting pointed to 7 enriched transcription factor binding motifs (Table S3, sheet B). In muscle, NRF1 was enriched for the genes contributing to enrichment of the three gene sets with lower expression in LBW vs NBW men after the control study (Table S3, sheet C), whereas 13 motifs were found for the genes contributing to the three KEGG pathways downregulated in LBW vs NBW men after fasting (Table S3, sheet D). Interestingly, 3 of these transcription factors were also differentially expressed in muscle from LBW vs NBW men based on  $p < 0.05$ , including *NRF1* that showed a higher expression in LBW men after the control study ( $p = 0.048$ ), and *ELK1* and *ELK3* that both

had higher expression in LBW vs NBW men after fasting (*ELK1*:  $p = 0.039$ ; *ELK3*:  $p = 0.009$ ; Table S3, sheet C and D).

### 3.4. Validation of gene expression differences in SAT from LBW vs NBW men using data from a different cohort

We have previously shown that a 5-day high-fat overfeeding (HFO) causes differential expression of >16% of all genes in SAT when combining data from 24 NBW and 16 LBW men [24]. In the present study, we used the SAT expression array data from our previous study and performed GSEA analyses to investigate if some of the KEGG gene sets that were differentially expressed in LBW vs NBW men in the fasting study were also different between the birth weight groups in the overfeeding study.

In line with the data presented in Fig. 2, OXPHOS and various other central metabolic pathways such as TCA cycle, pyruvate metabolism and biosynthesis of unsaturated fatty acids displayed lower expression in LBW vs NBW men after a control diet followed by overnight fast in this independent cohort (Fig. 3A and D; Fig. S1; Table S4, sheet C). Moreover, there was an increased expression of genes in several pathways related to the immune system in LBW vs NBW men – several of which were also upregulated in LBW vs NBW men after fasting in the present study (Fig. 2; Fig. S1; Tables S2 and S4). Interestingly, after the 5-day HFO diet, the expression of genes in metabolism pathways were no longer significantly different between the birth weight groups (Fig. S1; Table S4, sheet D). However, LBW men showed an upregulation of the antigen processing and presentation pathway and a downregulation of the ribosome pathway compared to NBW men in SAT after both control diet and HFO (Fig. S1; Table S4).



**Fig. 3.** Genes involved in oxidative phosphorylation have lower expression in subcutaneous adipose tissue and skeletal muscle from low vs normal birth weight men after control conditions and 5 days high-fat overfeeding, but not after 36 h fasting. These plots of log2 delta mRNA values show that OXPHOS genes ( $n = 142$ ) are lower in SAT (A, B, D) and skeletal muscle (A, C) from LBW vs NBW men during control conditions but higher in LBW vs NBW men after 36 h fasting (A, B, C). HFO, high-fat overfeeding; LBW, low birth weight; NBW, normal birth weight; OXPHOS, oxidative phosphorylation; SAT, subcutaneous adipose tissue.

Importantly, we were able to validate the expression differences seen in SAT between birth weight groups at control conditions in our fasting study using data from an independent cohort of LBW and NBW subjects using similar inclusion criteria.

### 3.5. Gene expression changes in SAT and muscle after fasting vs control studies reveal different response in individuals born with LBW compared with NBW

To investigate if LBW and NBW individuals had a different response to fasting regarding expression in SAT and muscle, we next compared the expression levels within each birth weight group after fasting compared to the control study. We only included samples with data from both time points (fasting and control study), resulting in 7 NBW and 8 LBW men in SAT, and 8 NBW and 8 LBW men in skeletal muscle, in the paired analyses of the transcriptional response to fasting vs control.

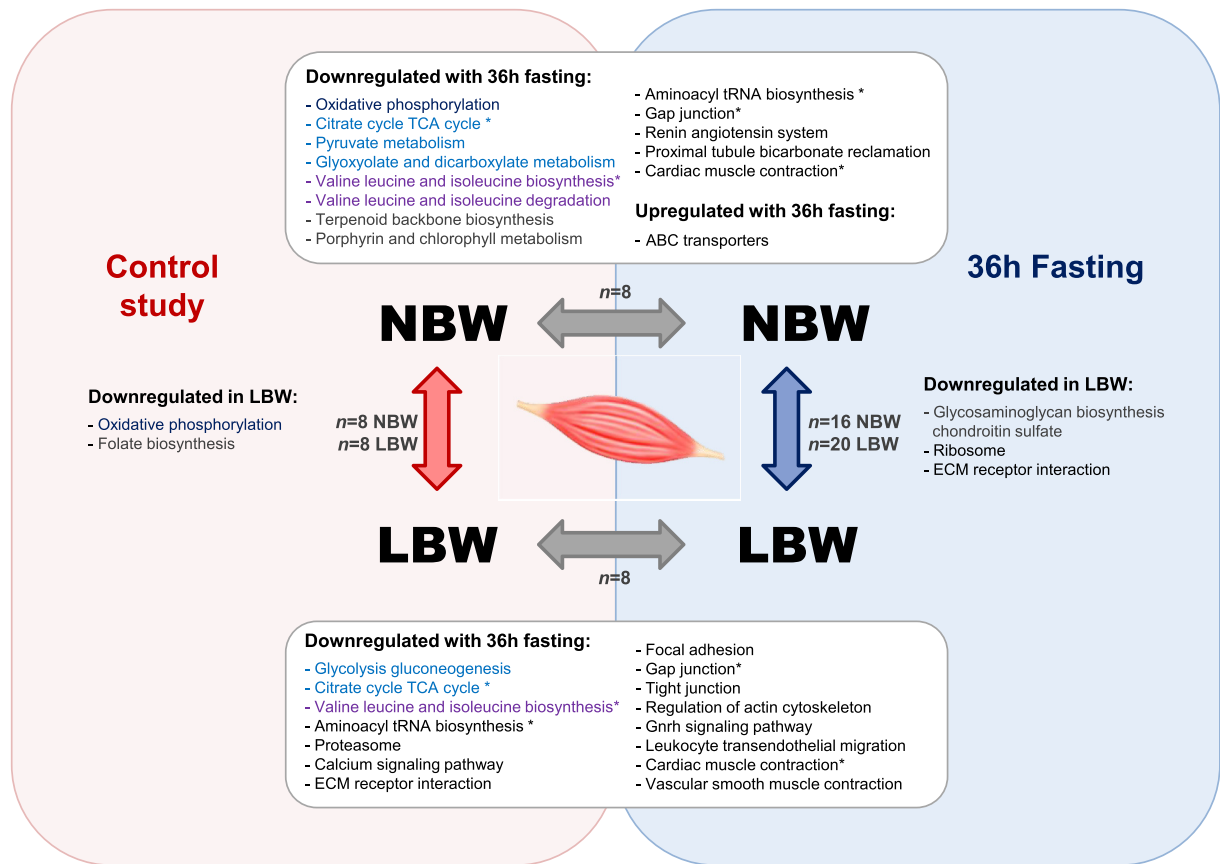
#### 3.5.1. SAT gene expression

First, we performed a GSEA pathway analysis, here based on data from paired analyses of all transcripts in the control study compared with fasting. In SAT from NBW subjects, 20 KEGG pathways were downregulated and none were upregulated after fasting compared with the control study (Fig. 2; Table S2, sheet E). These were mainly involved in metabolism, including OXPHOS, carbohydrate, lipid, amino acid and nucleotide metabolism (Fig. 2; Table S2, sheet E). To visualize how specific genes in a metabolic gene set were regulated with fasting in the two groups, we plotted gene expression fold changes (log2) for all OXPHOS genes in LBW and NBW men (Fig. 5A). Here, it was clear that

the same genes were regulated differently in SAT from LBW and NBW individuals. In SAT from LBW men, no metabolic pathways were significantly lower after fasting compared to the control study, and among the 14 pathways upregulated after fasting, two involved metabolism (Fig. 2; Table S2, sheet F). Interestingly, while the proteasome pathway was significantly upregulated in SAT from LBW men with fasting, it was downregulated in SAT from NBW men with fasting (Fig. 2; Table S2, sheet E and F). Hence, the genome-wide expression changes in response to fasting were very different in SAT from LBW vs NBW men (Fig. 2; Fig. 5D).

#### 3.5.2. Muscle gene expression

The gene expression changes seen in response to fasting based on GSEA were more similar between LBW and NBW men in skeletal muscle than in SAT. In muscle from NBW individuals, 13 KEGG pathways were significantly downregulated in response to fasting vs control study and most of them were related to metabolism (Fig. 4; Table S2, sheet G). Moreover, ABC transporters (involved in membrane transport) was upregulated with fasting in NBW men (Fig. 4; Table S2, sheet G). Similar to SAT, OXPHOS was the pathway that was most significantly downregulated with fasting in muscle from NBW men. When plotting the OXPHOS gene expression fold changes (log2) in LBW and NBW men (Fig. 5B), it was again clear that the OXPHOS response to fasting was different in muscle from LBW and NBW men. In muscle from LBW men, 15 pathways were downregulated after fasting vs control study. Here, many pathways were involved in cellular processes, but also metabolism (Fig. 4; Table S2, sheet H). Of note, five pathways were significantly downregulated in muscle from both LBW and NBW subjects after fasting vs control study, e.g. the TCA cycle, GAP junction,



**Fig. 4.** Gene sets with significantly different mRNA expression in skeletal muscle after 36 h fasting vs control study (horizontally) and in LBW vs NBW men (vertically). Metabolism KEGG pathways are coloured by subgroup: Energy (dark blue), Carbohydrate (light blue), Amino acid (purple), Glycan biosynthesis, Cofactors and vitamins and Terpenoids and polyketides (grey). Human diseases pathways are not included in the figure. \*Pathway regulated in the same direction in response to fasting in LBW and NBW men. LBW, low birth weight; NBW, normal birth weight.

aminoacyl tRNA biosynthesis and cardiac muscle contraction (Fig. 4; Table S2, sheet G and H). Taken together, we found different transcriptional responses to fasting for some gene sets in skeletal muscle from LBW vs NBW men (Fig. 5E), but it was not as pronounced as in SAT (Fig. 5D).

We proceeded to study expression changes of individual genes. In both SAT and skeletal muscle we found thousands of nominal associations ( $p < 0.05$ ). After correction for multiple testing ( $FDR < 5\%$ ,  $q < 0.05$ ), in skeletal muscle we found 20 transcripts that changed significantly with fasting in LBW men, while 134 transcripts changed with fasting in NBW men (Table S5). Four transcripts changed in the same direction (all upregulated) in both groups, representing *YPEL1* (LBW fold change (FC) 1.49, NBW FC 1.65), *MAML3* (LBW FC 1.26, NBW FC 1.34), *LMTK2* (LBW FC 1.30, NBW FC 1.27) and *ZFAND5* (LBW FC 1.46, NBW FC 1.48).

### 3.6. In contrast to fasting, there are similar expression changes in SAT in response to 5 days high-fat overfeeding in LBW and NBW men

To investigate if the discrepancy in the SAT gene expression response in LBW vs NBW men was specific to fasting or also applied to other metabolic challenges, we performed new analyses of a SAT mRNA expression data set from the independent LBW and NBW overfeeding cohort mentioned above [24]. In this previous study, 16 LBW men and 24 NBW men were exposed to 5 days of HFO and a control diet in a randomized order, and mRNA expression was analysed in SAT biopsies that were excised after overnight (12 h) fasting after both diets.

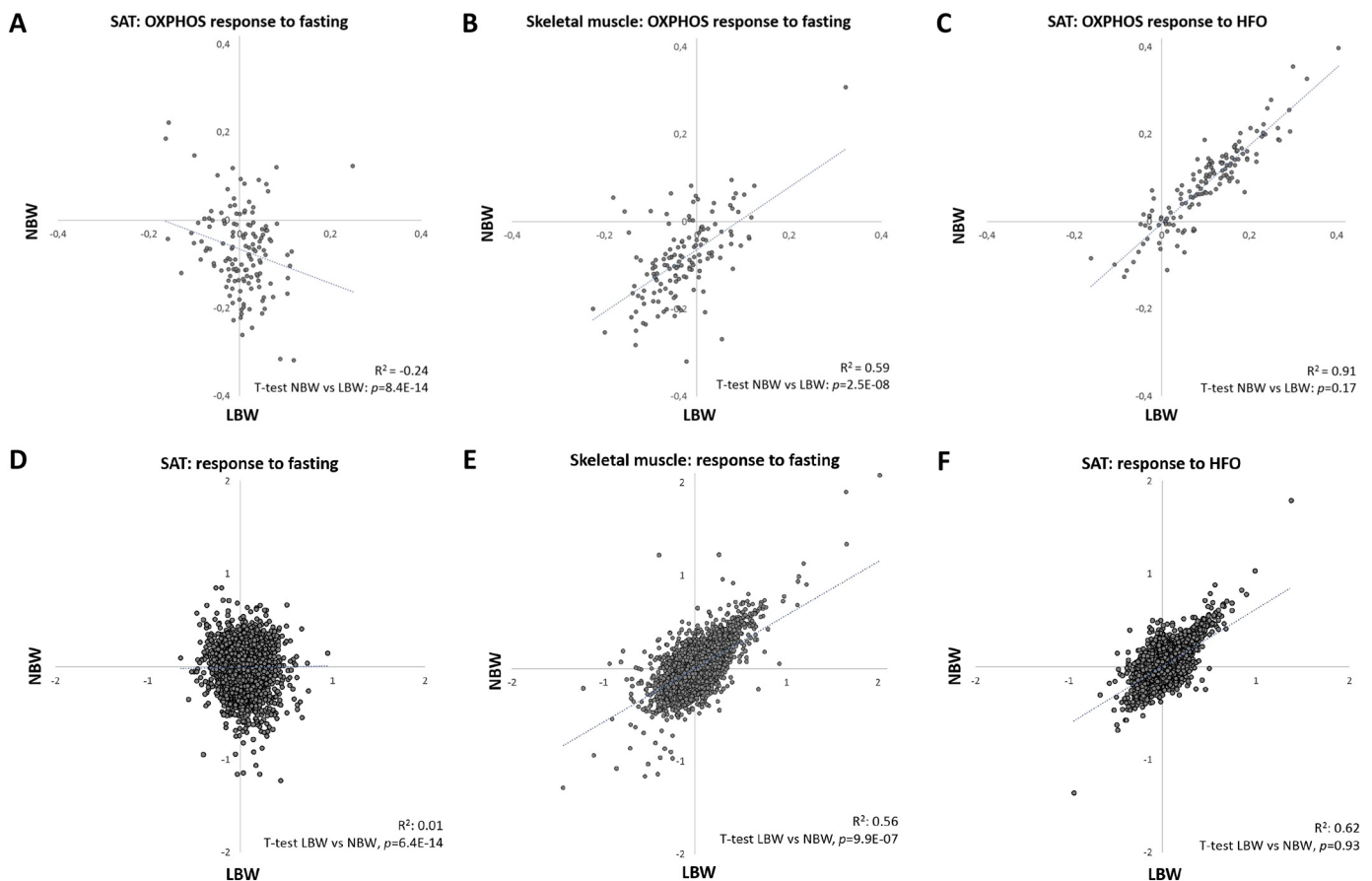
In contrast to the results seen after fasting (Fig. 2), based on GSEA analyses, 5 days HFO induced similar gene expression changes in SAT

from LBW and NBW men (Fig. S1; Table S4, sheet A and B). OXPHOS, TCA cycle, pyruvate metabolism, biosynthesis of unsaturated fatty acids and proteasome were among the most significantly upregulated KEGG gene sets after HFO in both birth weight groups, and among the 24 significantly upregulated KEGG pathways in NBW men, 23 were also significantly upregulated in the LBW men (Table S4, sheet A and B). Interestingly, among these 24 pathways upregulated in SAT by HFO in NBW men, 13 were downregulated in NBW men in response to fasting (Table S4, sheet B), whereas none of the 40 upregulated pathways by HFO in LBW men were significantly downregulated by fasting. Only one pathway was significantly downregulated in response to HFO in the LBW (ribosome) and one in the NBW (ether lipid metabolism) men, respectively. By plotting fold changes for all OXPHOS genes, it was clear that these genes were regulated similarly in LBW and NBW men (Fig. 5C). A highly significant correlation between all transcriptional changes in SAT ( $R^2 = 0.62$  for all 28,829 transcripts) to the HFO challenge, with similar changes in both groups based on a  $t$ -test ( $p = 0.93$ ), was seen in NBW and LBW men (Fig. 5F), in contrast to the transcriptional response to 36 h fasting in SAT ( $R^2 = 0.01$ ,  $t$ -test:  $p = 0.6E-14$ ; Fig. 5D). These data demonstrate that the transcriptional changes in SAT in response to HFO are similar between LBW and NBW men, while LBW respond transcriptionally differently than NBW men to fasting.

### 3.7. Few DNA methylation differences in genes with different expression in LBW vs NBW men after control and fasting studies

Epigenetic alterations have been suggested as potential mechanisms for how an impaired in utero environment may influence risk of metabolic disease in later life [16,28]. Hence, in the search for molecular





**Fig. 5.** Men born with low vs normal birth weight have different transcriptional responses to 36 h fasting in adipose tissue and skeletal muscle but similar response to 5 days high-fat overfeeding in adipose tissue. These plots of log2 delta mRNA values show that OXPHOS genes (A–C;  $n = 142$ ) and all array transcripts (D–F;  $n = 28,829$ ) are regulated differently with fasting in SAT, and partly in muscle, in LBW and NBW men, and that the mRNA response to overfeeding in SAT is very similar. HFO, high-fat overfeeding; LBW, low birth weight; NBW, normal birth weight; OXPHOS, oxidative phosphorylation; SAT, subcutaneous adipose tissue.

mechanisms explaining the different gene expression responses to fasting between LBW and NBW men, we investigated the level of DNA methylation of selected genes and regions based on significant expression differences.

### 3.7.1. SAT DNA methylation

First, we extracted a list of genes which expression levels were driving the significant enrichment for downregulation of the OXPHOS pathway seen in SAT from LBW vs NBW men after the control study, which were also driving the same pathways to be upregulated in LBW vs NBW men after fasting (Table S2, sheet A and B). We next extracted DNA methylation data from the sites annotated to the 39 OXPHOS genes that fulfilled this criterion (735 CpG sites). Among these, we found nominally differential methylation of 27 CpG sites ( $p < 0.05$ ) in SAT from LBW vs NBW men after the control study, and of 34 CpG sites after 36 h fasting (Table S6, sheet A). These epigenetic differences were not significant after correction for multiple testing ( $q < 0.05$ ) and none of the CpG sites did overlap, i.e. they were only differentially methylated ( $p < 0.05$ ) after the control study or fasting in SAT from LBW vs NBW men. However, although in different genomic positions, a total of 17 of the selected OXPHOS genes showed differential methylation in both groups.

Secondly, we extracted methylation data for CpG sites annotated to the genes driving the KEGG proteasome pathway that was downregulated in SAT from NBW but upregulated in SAT from LBW men after fasting vs control study (Fig. 2; Table S2, sheet E and F). For the 12 genes driving the enrichment of the proteasome pathway, we found nominally altered methylation ( $p < 0.05$ ) after fasting vs the control

study of 11 CpG sites in NBW and 7 CpG sites in LBW men (Table S6, sheet B); none of them did overlap between the groups. Most of these sites were annotated to *PSMD7*, *SIRT3/PSMD13* and *PSME3* (four, three and three CpG sites, respectively). These results suggest that the impact of DNA methylation on the differential gene expression in response to fasting in SAT from LBW vs NBW men is modest.

### 3.7.2. Skeletal muscle DNA methylation

Finally, we investigated DNA methylation for CpG sites annotated to the individual genes in muscle that significantly ( $q < 0.05$ ) changed expression after fasting vs control study, from LBW and NBW men, respectively (Table S5). In the LBW men, 20 individual genes were differentially expressed in muscle after fasting vs control study with  $FDR < 5\%$ . Annotated to 17 of these genes, we found 18 CpG sites with nominally differential methylation in muscle from LBW men after fasting vs control study ( $p < 0.05$ ) (Table S6, sheet C). These include *YPEL1*, *MAML3*, *LMTK2* and *ZFAND5*, which show expression changes in muscle in both LBW and NBW men after fasting vs control study. In NBW men, 67 CpG sites annotated to 92 individual genes with altered muscle expression after fasting vs control study (with  $q < 0.05$ ) also displayed nominal differences in methylation with  $p < 0.05$  (Table S6, sheet B). Here, five CpG sites were annotated to *MAML3* and two to *YPEL1*.

Although some epigenetic differences were found between the birth weight groups and in response to fasting, the magnitude and significance of these changes were very subtle, unlike the clearly differential gene expression changes detected in LBW vs NBW men.

#### 4. Discussion

This study reveals that healthy young men born at term with LBW have a different transcriptional response to fasting in SAT and, to a lesser degree, in skeletal muscle, compared to men born with NBW. Interestingly, after an isocaloric diet and a regular overnight fast (the control study), SAT of LBW men showed lower expression of genes involved in OXPHOS and other pathways for essential carbohydrate (e.g. glycolysis/gluconeogenesis, TCA cycle, pyruvate metabolism), amino acid (e.g. beta alanine metabolism, glutathione metabolism, valine leucine and isoleucine degradation) and lipid (e.g. biosynthesis of unsaturated fatty acids) metabolism compared with NBW men. Importantly, the lower expression of these and other metabolism pathways in SAT from LBW vs NBW men was replicated in an independent study. Also in skeletal muscle, the OXPHOS gene set was significantly downregulated in LBW vs NBW men after the control study. Notably, these healthy young LBW men showed a similar phenotype of OXPHOS gene sets downregulated in both muscle and SAT to subjects with overt type 2 diabetes [22,25]. Type 2 diabetes is a multifactorial and polygenic disease where small alterations and/or effect sizes of many genes contributing to the disease. Indeed, several studies analysing gene expression in tissues from individuals with type 2 diabetes used GSEA to find expression differences in gene sets rather than focusing on individual genes [22,25,29]. The same applies to the present study; we found more significant expression differences of gene sets than of individual genes.

In response to 36 h of fasting, OXPHOS and metabolism gene sets were *downregulated* in SAT from NBW men, whereas none of these gene sets changed significantly in SAT from LBW men. A similar pattern was seen in skeletal muscle with the OXPHOS gene pathway downregulated by fasting in the NBW men only. Consequently, and paradoxically, the LBW men ended up, after fasting, having *higher* SAT expression of essential metabolism gene sets including OXPHOS, glycolysis/gluconeogenesis and beta alanine metabolism compared with the NBW men. Interestingly, the LBW men in this study had significantly lower energy expenditure (EE) and fat oxidation (FOX) compared to the NBW men during fasting [11]. Reduced EE and FOX suggest a lower capacity for mitochondrial oxidation of fatty acids in LBW men, which is reflected by our gene expression results of metabolic pathways in both skeletal muscle and SAT during the control (overnight fasting only) study.

Previous studies have suggested that the level of energy metabolism may regulate mRNA turnover/degradation [30]. Hence, the decreased energy metabolism in LBW men compared with the NBW control group during fasting, could potentially be associated with relatively decreased mRNA turnover/degradation [30]. This is one hypothetical explanation to the otherwise paradoxical finding of reduced *in vivo* energy metabolism in the face of increased gene expression levels of metabolic genes in SAT from LBW subjects during fasting. In further support of this hypothesis, an association between mRNA turnover/degradation and mitochondrial oxidative capacity (mitochondrial content) has been reported in striated muscle fibers [31]. Similarly, another study showed that decreased expression of a glucose transporter gene was regulated at the mRNA degradation step in response to decreased glycolytic flux caused by mutations in other genes of the glycolysis pathway [32]. However, future studies are needed to further dissect this hypothesis.

Epigenetic programming associated with an adverse foetal development could explain why LBW men respond transcriptionally different to fasting. We studied DNA methylation differences in genes that were differentially expressed in both SAT and muscle, but we only found modest methylation differences between LBW and NBW groups. Furthermore, few and only minor changes of DNA methylation induced by fasting were detected in the CpG sites studied. Nevertheless, among the few genes showing both differential DNA methylation as well as gene expression levels in the LBW vs NBW men in this study, some have

previously been found to be associated with type 2 diabetes including *COX7A1*, *COX7A2* and *UQCRB* [22,29,33]. In some of our previous studies, we found significant DNA methylation differences in LBW vs NBW men [15,24,34]. For example, in SAT biopsies from the validation cohort (HFO study), we found 53 CpG sites with significant DNA methylation differences in LBW vs the NBW men after either control or overfeeding diets, with absolute methylation differences of up to 13 percentage points between groups [24]. Importantly, in another of our previous studies, substantial epigenetic differences were detected in adipose progenitor cells from LBW vs NBW men, suggesting that epigenetic differences in stem cells play a more important role than in mature SAT [34]. This may explain why we do not see more impressive epigenetic differences between birth weight groups in the current work. It should also be noted that the methylation array only covers ~1.5% of the CpG sites of the human methylome and that other CpG sites, not analysed here, may be affected. Interestingly, Kazachenka et al. recently used whole-genome bisulfite sequencing and RNA sequencing to analyse the whole methylome and transcriptome in rodents and they found that retrotransposons with variable DNA methylation only rarely resulted in variable expression of adjacent genes, suggesting that DNA methylation at repetitive elements most often do not cause phenotypic effects [35]. In line with this, other epigenetic mechanisms such as histone modifications and non-coding RNAs might have a larger contribution to the differences in mRNA levels and responses to fasting in our study of LBW vs NBW men, which should be further investigated.

In addition to the differences in OXPHOS and carbohydrate metabolism gene sets during control conditions, LBW subjects also showed lower SAT expression of gene sets involved in glutathione metabolism, beta alanine metabolism, and valine, leucine and isoleucine degradation. These differences were seen consistently in the same overnight fasted control conditions the validation cohort. Fasting induced a downregulation of these pathways in the NBW men only. Interestingly, we recently showed that LBW men exhibit disproportionately elevated plasma alanine, proline, methionine, citrulline as well as total amino acid levels when exposed to a 5-days high-fat overfeeding challenge compared with NBW men [36–38]. It may be speculated that reduced expression of genes involved in amino acid metabolism in adipose tissue, as well as potentially other organs, to some extent may contribute to elevated plasma amino acid levels in LBW subjects when exposed to overfeeding. Further studies are needed to confirm or reject this hypothesis.

We used PSCAN and JASPAR bioinformatical tools to identify enriched transcription factor motifs in genes that are differentially expressed in LBW vs NBW men. Interestingly, *KLF14* was among the transcription factors with a significantly enriched binding motif for the core genes that were driving pathways with significantly lower expression in SAT from LBW vs NBW men after control conditions. *KLF14* has been reported to be involved in the development of type 2 diabetes in humans and mice studies [39,40]. To this end, increased age was associated with increased DNA methylation of *KLF14* in human adipose tissue, liver, pancreatic islets and blood [12,41,42]. Furthermore, increased methylation of *KLF14* was associated with decreased future risk of type 2 diabetes and increased insulin secretion [12].

By performing GSEA analyses on SAT gene expression data from LBW and NBW individuals in one of our previous studies [24], we validated our findings of lower gene expression of gene sets involved in OXPHOS, TCA cycle and pyruvate metabolism, among others, in LBW vs NBW men. Interestingly, we also found that the transcriptional response to 5-days high-fat overfeeding (HFO) in SAT was very similar in both birth weight groups. In fact, of the 24 pathways that were significantly upregulated in the LBW men with HFO, 23 were also significantly upregulated in the NBW men, including all 17 metabolism pathways, and no metabolism pathways were significantly different between LBW and NBW groups after the 5-day HFO diet. Thus, foetal growth does not seem to affect the SAT transcriptional response to

high-fat / high-calorie diets to the same degree as fasting. While half of the pathways that were upregulated by HFO in SAT were also downregulated by fasting in NBW men, none of the metabolic pathways changed in opposite directions with fasting and HFO in LBW men. Possible explanations to why LBW men are not downregulating metabolic pathways during fasting could be that their adipose tissue has a different degree of inflammation/infiltration of immune cells after fasting. Immune system pathways including hematopoietic cell lineage, natural killer mediated cytotoxicity, chemokine signaling and T- and B-cell receptor pathways were upregulated in SAT from LBW vs NBW men after fasting, as well as after overnight fasting in the validation cohort. Infiltrating macrophages have the capability to secrete inflammatory cytokines and it is speculated that this is a contributing mechanism for insulin resistance [43]. It is possible that the expression differences in LBW vs NBW men predispose to insulin resistance and type 2 diabetes. Indeed, we have previously shown that the OXPHOS pathway and numerous of the metabolic pathways downregulated in SAT of LBW men also are downregulated in subjects with type 2 diabetes compared with non-diabetic controls [25]. In the same study, we found upregulation of immune system pathways in SAT of diabetic subjects, in line with what we find already in young healthy LBW men in this study.

In summary, this study supports the concept of developmental programming with early life conditions influencing transcriptional fingerprints in insulin sensitive tissues. Importantly, transcription of gene sets involved in metabolism, and energy metabolism in particular, and to some degree also the epigenetic methylation patterns, are different in SAT and muscle from LBW vs NBW young adults already during control conditions and are downregulated in response to a fasting challenge only in the NBW men. These data provide further evidence of an adverse intrauterine environment to play a key role in the development of metabolic disease including type 2 diabetes.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2019.08.017>.

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## Author contributions

S.W.J. and A.V. designed the clinical study. L.G., T.R., L.H., E.N., C.B. and C.L. contributed to the design of the study and the experiments. L.G., T.R., S.W.J., A.P., L.H., E.N., C.B., A.V. and C.L. performed the research and analysed data. All authors interpreted and discussed the data. L.G., T.R., A.L., and C.L. wrote the manuscript. All authors reviewed and revised the manuscript critically. L.G. and C.L. are the guarantors of this work and, as such, have full access to all the study data and take responsibility for the integrity of the data.

## Declaration of Competing Interest

None.

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